Multilayered Polycaprolactone/Gelatin Fiber-Hydrogel Composite for Tendon Tissue Engineering

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Abstract

Regeneration of injured tendon and ligament (T&L) remains a clinical challenge due to their poor intrinsic healing capacity. Tissue engineering provides a promising alternative treatment approach to facilitate T&L healing and regeneration. Successful tendon tissue engineering requires the use of three-dimensional (3D) biomimetic scaffolds that possess the physical and biochemical features of native tendon tissue. We report here the development and characterization of a novel composite scaffold fabricated by co-electrospinning of poly- ϵ -caprolactone (PCL) and methacrylated gelatin (mGLT). We found that photocrosslinking retained mGLT, resulted in a uniform distribution of mGLT throughout the depth of scaffold and also preserved scaffold mechanical strength. Moreover, photocrosslinking was able to integrate stacked scaffold sheets to form multilayered constructs that mimic the structure of native tendon tissues. Importantly, cells impregnated into the constructs remained responsive to topographical cues and exogenous tenogenic factors, such as TGF- β 3. The excellent biocompatibility and highly integrated structure of the scaffold developed in this study will allow the creation of more advanced tendon graft that possesses the architecture and cell phenotype of native tendon tissues.

1. Introduction

Tendons and ligaments are prone to injuries such as rupture and laceration due to their load-bearing nature [1, 2]. In cases of severe tendon injury, surgical intervention is employed to repair or replace the damaged tendon with autografts, allografts, xenografts, or prosthetic devices [3-5], for the natural healing process is slow and insufficient [6, 7]. To date, the clinical outcomes of tendon repair remain limited and unsatisfactory due to donor site morbidity, high failure rates, risk of injury recurrence, and limited long-term function restoration [8-10]. These limitations have spurred the development of tendon tissue engineering approaches, which apply combination of cells, scaffolds and bioactive molecules, as a promising strategy to create functional tissue replacements or to enhance the innate healing process [11, 12]. Ultimately, tendon tissue engineering aims at improving the quality of healing in order to fully restore tendon structure and function [13].

Tendon tissues are composed of densely packed aligned collagen fibrils that connect muscle to bone [7, 14]. Therefore, aligned nano- and micro-fibrous scaffolds fabricated by electrospinning have been extensively explored in attempts to recapitulate the mechanical and topographical characteristics of native tendon tissue [15-17]. Electrospun poly- ϵ -caprolactone (PCL) scaffolds are frequently used in tendon tissue engineering as well as applications for other soft tissues. PCL is an aliphatic linear polyester approved by the U.S. Food and Drug Administration for clinical use [18]. It is biocompatible, bioresorbable and a low-cost synthetic polymer. Of equal importance, PCL exhibits low degradation rate due to its semi-crystalline and hydrophobic

nature [19, 20], making it a suitable graft material to facilitate the relatively slow healing process of injured tendons [21, 22]. However, the hydrophobic nature of PCL often results in poor wettability [23], inadequate cell attachment, and poor tissue integration [24] when used in tissue engineering. Moreover, as a synthetic polyester, its lack of bioactivity is a major challenge for PCL to direct cell behavior after seeding due to the absence of cell-binding motifs found in natural extracellular matrix (ECM) proteins [25].

Hydrogels prepared from collagen and its derivative, gelatin, represent another class of scaffolds for regenerating and repairing a wide variety of tissues and organs [26, 27]. Unlike other types of scaffolds, hydrogels retain a large volume of water and thus provide a highly hydrated environment similar to that in native tissues. Cells encapsulated within collagen/gelatin hydrogels can be easily distributed homogeneously by simple mixing during gel preparation [28, 29]. Importantly, collagen and gelatin, as constituents of natural ECM, better mimic at least in part the native tissue microenvironment, as compared to synthetic polymers [30, 31].

Nevertheless, improvement in the mechanical properties and introduction of topographical cues are needed to apply these hydrogels to tendon grafts that aim at reproducing the mechanical and structural features of native tendon tissues.

The organization of native ECM may be viewed as a cell-containing hydrogel reinforced by structural fibers. An engineered scaffold consisting of hydrogels and electrospun fibers may thus be considered as a biomimetic of the ECM. For example, a microfiber-reinforced silk hydrogel displayed a greatly improved modulus compared to a fiber-free hydrogel [32]. In addition, hydrogels composed of natural proteins could provide the bioactive motifs absent from synthetic polymeric scaffolds to enhance control of cell binding and fate determination [28, 33]. In terms of tendon tissue engineering, an ideal composite scaffold consisting of hydrogel and fibrous

scaffold has yet to be developed. Consequently, little is known about the effects such a composite scaffold may have on the activities of encapsulated cells.

In this study, we have developed a novel composite scaffold as a tendon graft consisting of electrospun PCL microfibers and methacrylated gelatin (mGLT). We have optimized the retention of mGLT by photocrosslinking and its integration with the fibrous scaffold.

Simultaneous cell seeding and photocrosslinking between scaffold layers were performed to create cell-impregnated multilayered constructs, and their mechanical properties and architecture and the activity of encapsulated cells were assessed. Our results show that this novel cell-scaffold construct combines the advantages of PCL nanofibrous scaffolds and gelatin hydrogels to mimic the mechanical feature, structure and cell phenotype of native tendon tissue.

2. Materials and methods

2.1. Synthesis of methacrylated gelatin

Methacrylated gelatin (mGLT) was synthesized using an established protocol with slight modification [29, 34]. Gelatin (GLT, Sigma-Aldrich) was dissolved in deionized H₂O at 37 °C (30%, w/v). Methacrylic anhydride (Sigma-Aldrich) was then added dropwise into the mixture at 37 °C under mild agitation to react with amine groups on GLT for 24 hours (Supplementary Fig. S1 A). Reacted mGLT solution was dialyzed against water to completely remove low molecular-weight byproducts using 3,500 NMWCO dialysis cassettes (Thermo Scientific). Dialyzed mGLT was lyophilized and stored desiccated for future use. The methacrylation rate of the product was ~80% [34]. The visible light (VL)-activated photo-initiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as described by Fairbanks et al. [35].

2.2. Fabrication of composite scaffolds

Composite scaffolds containing interspersed PCL and mGLT fibers were produced using dual electrospinning [36]. PCL particles (70k-90k, Sigma-Aldrich) was dissolved in 2,2,2-trifluoroethanol (TFE, Sigma-Aldrich, 18% w/v). Dehydrated mGLT was dissolved in 95% TFE (20% w/v). Each polymer solution was extruded through a 22-gauge blunt-tip needle at 2 mL/h for 1 hour. The spinnerets were charged with an optimized DC potential (8 kV for PCL and 15 kV for mGLT, respectively) and aligned in opposing positions on each side of the collection mandrel with a distance of 15 cm between the needle tip and mandrel (Fig. 1). The scaffold was stored desiccated under vacuum to remove residual solvent. To retain mGLT in the composite scaffold, 0.5% LAP solution was cast onto dry composite scaffold surface at 15 $\mu\text{L}/\text{cm}^2$ and allowed to spread until the scaffold was completely wet. LAP was then activated by exposure to VL irradiation (450-490 nm) to photocrosslink the methacrylate groups of the dissolved gelatin in the scaffold (Fig. 1, Supplementary Fig. S1 B).

2.3. Imaging of composite scaffolds

Polymers were fluorescently labeled to track the presence and interspersions of the two distinct fiber populations: PCL solution was mixed with 0.1% (v/v) Vybrant® DiI Cell-Labeling Solution (DiI, Life Technologies), and non-methacrylated GLT was conjugated with fluorescein 5(6)-isothiocyanate (FITC, Sigma-Aldrich) before dissolution, respectively. Fibers were dual-electrospun onto glass slides for 5 min and imaged before and after wetting using an Olympus CKX41 inverted fluorescent microscope equipped with a CCD camera. Additionally, scaffold

surface was examined by scanning electron microscopy (SEM, field emission, JEOL JSM6335F) operated at 3 kV accelerating voltage and 8 mm working distance.

2.4. Histological examination of composite scaffolds

Picrosirius red staining was employed to assess gelatin retention and distribution within scaffolds. Composite scaffolds before and after photocrosslinking were washed in PBS at 37 °C overnight under mild agitation, frozen-embedded in OCT compound (4583 Scigen Scientific), and cryosectioned at 15 µm thickness using a Leica CM 1850 cryotome. Sections were washed in PBS and stained with 0.1% sirius red in saturated picric acid (Electron Microscopy Sciences) for 1 hour. To visualize impregnated cells, cryosections of fixed, cell-seeded scaffolds were incubated with ethidium homodimer-1 (EthD-1, Life Technologies) to label cells via DNA binding.

2.5. Biochemical composition of composite scaffolds

Gelatin content of composite scaffolds was quantified by the Chloramine-T based hydroxyproline assay (Fisher Scientific and Sigma-Aldrich). Reaction product was measured spectrophotometrically at 550 nm using a microplate reader (BioTek). Relative gelatin retention rate was calculated as the reading of washed scaffold divided by that of dry scaffold.

2.6. Cell isolation and culture

Human adipose-derived stem cells (hASCs) were obtained from lipoaspirate-derived fat tissue of two donors (34 years old male and 38 years old female) using an automated cell isolation system (Tissue Genesis). The protocol was approved by the Institutional Review Board of the University of Pittsburgh. Isolated hASCs were cultured in growth medium (GM) consisting of DMEM-high

glucose (Gibco), 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (P/S, Gibco). Multipotency was confirmed by established differentiation tests [37].

2.7. Creation of multilayer constructs

Scaffold pairs were prepared by overlaying one third the area of a rectangular scaffold piece atop another scaffold piece of identical shape. The scaffold pair was then wetted with photo-initiator and crosslinked by VL irradiation (Fig. 4 A), followed by gentle washing in PBS at 37 °C for up to 7 days. To create multilayered structures, 5 sheets of scaffold of identical rectangular shape were wetted with photo-initiator solution, stacked, and exposed to VL for 1 min on each side to crosslink adjacent scaffold layers (Fig. 5 A). For these multilayer constructs, photo-initiator was dissolved in 8% mGLT solution to further reinforce the association between scaffold layers.

Sandwich constructs made from alternative layers of fibrous PCL and mGLT solution were also prepared and subjected to the same photocrosslink protocol. After 7 days of washing in PBS at 37 °C, scaffold integrity was examined by picrosirius red staining of orthogonal cryosections.

2.8. Mechanical testing

Tensile properties of single layer scaffold and multilayer constructs were analyzed by using the Bose 3230 mechanical tester. Samples were securely mounted and loaded with uniaxial force applied at a displacement rate of 0.2 mm/s until 10 mm displacement. The tensile force and the displacement were recorded. The slope of the linear portion of the stress–strain curve was calculated as the elastic modulus.

2.9. Creation of a tendon-mimetic construct by multilayer scaffolds

Aligned composite scaffolds were fabricated by dual electrospinning. hASCs were detached and suspended in LAP-containing 8% mGLT solution and cast onto scaffold sheets for hydration before VL irradiation. Multilayer constructs consisting of 5 sheets of cell seeded, aligned scaffolds were then prepared as described above. To induce tenogenic differentiation, constructs were maintained for 7 days in differentiation medium (DM) consisting of DMEM, 2% FBS, P/S, and 10 ng/ml TGF- β 3 (PeproTech).

2.10. Cell proliferation tests

Cells encapsulated in multilayer constructs were maintained in standard GM and subject to MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) at 1 day or 7 days after initial seeding. A_{490} was determined spectrophotometrically using a microplate reader (BioTek). Double stranded DNA (dsDNA) content in each group was determined fluorimetrically by Picogreen assay (Quant-iT PicoGreen, Invitrogen; excitation 480 nm, emission 520 nm). All readings were normalized to the cell free control group at each time point.

2.11. Cytoskeleton fluorescent staining

Cell seeded, single layer scaffold sheets were washed in PBS, fixed in 4% paraformaldehyde and incubated with 1% bovine serum albumin (BSA). Cells were permeabilized in 0.1% Triton X-100 for 5 min and then incubated with phalloidin for 30 min at room temperature (Alexa Fluor 488 phalloidin, Life Technologies). Lastly, cells were rinsed with PBS, nuclear counterstained with DAPI (Life Technologies), and imaged using a confocal microscope (Olympus FluoView 1000).

2.12. Real-time PCR analysis

Total cellular RNA was isolated using an RNA extraction Kit (Qiagen) and first-strand cDNA synthesized with random hexamer primers (SuperScript III First-Strand cDNA synthesis kit, Invitrogen). Quantitative real-time PCR was performed using SYBR green Supermix in a Step One Plus real-time PCR system (Applied Biosystem, Life Technologies) to analyze expression of tenogenic markers, scleraxis (SCX) and tenascin-C (TNC). The targets and sequences of primers are shown in Table 1. The relative level of each gene was normalized to that of 18S rRNA and calculated using the $\Delta\Delta$ -Ct method.

2.13. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). All quantitative assays were performed for no less than three times independently (n equals to the number of samples tested). One-way ANOVA with Bonferroni post hoc tests and Student's t-test was performed to determine statistical significance. Significance was considered at $p < 0.05$.

3. Results

3.1. Organization of fibers in composite scaffold

Microscopic observation of the co-electrospun scaffold showed that the fluorescently labeled fibers were interspersed within the dry scaffold (Fig. 2 A, Composite Dry, red: PCL; green: mGLT). Hydration of the scaffold in an aqueous environment resulted in rapid dissolution of mGLT fibers, whereas PCL fibers remained intact (Fig. 2 A, Composite Wet, red: PCL; green: mGLT). SEM revealed a fraction of fibers with distinct ribbon-like morphology, in addition to the cylindrically shaped fibers seen in the dry composite scaffold mesh (Fig. 2 B, Composite Dry, indicated by arrow in insert). After hydration, the ribbon portion was no longer detectable; instead, adjacent fibers were found bridged or bundled by deposited sheaths (Fig. 2 B,

Composite Wet, indicated by arrows in insert). The alteration in scaffold organization is likely caused by the dissolution and deposition of incorporated gelatin during hydration, due to its high aqueous solubility. In contrast, scaffold made only of PCL showed no observable difference between the dry and wet state, exhibiting uniform shaped fibers without fused contacts (Fig. 2 B, PCL Dry vs. PCL Wet).

3.2. Biochemical and mechanical analysis of composite scaffold

Hydrated composite scaffold was exposed to LV irradiation for crosslinking of mGLT in the presence of photo-initiator (Fig. 1). Picrosirius staining of gelatin showed a pronounced difference in gelatin retention between non-crosslinked and crosslinked scaffolds (Fig. 3 A). Without crosslinking, the scaffold displayed little red staining, indicating loss of the majority of incorporated gelatin after washing (Fig. 3 A, Composite non-crosslinked). In contrast, a large amount of gelatin was retained and evenly distributed within the crosslinked scaffold mesh (Fig. 3 A, Composite crosslinked, red), suggesting excellent integration between gelatin and PCL fibers. The control consisting of mGLT solution cast atop PCL scaffold resulted in a distinct boundary between gelatin hydrogel and PCL, suggesting separation of the two scaffold formats (Supplementary Fig. S2).

Scaffolds were dried in an oven and weighed before and after gentle washing in PBS for 1 day at 37 °C to estimate the gelatin loss (Fig. 3 B, $p < 0.01$). After washing, non-crosslinked scaffolds lost one third of their initial weight ($32.87\% \pm 3.89$, $n=5$), whereas crosslinked scaffolds lost only 16% ($15.89\% \pm 3.50$, $n=5$). This finding is consistent with the gelatin content estimated by hydroxyproline assay: (Fig. 3 C, $p < 0.01$) crosslinked scaffold retained half of its initial gelatin content ($44.53\% \pm 1.58$, $n=4$), while non-crosslink scaffold preserved less than 3%.

(2.93% $\pm$ 0.46, n=4). We then analyzed the impact of crosslinking on the mechanical strength of scaffolds, and found a 16.0% reduction in maximum load of non-crosslinked material after washing. In contrast, crosslinked scaffold demonstrated consistent maximum load with no significant alteration compared to dry composite scaffold (Fig. 3 D).

3.3. Multilayered construct created by photocrosslinking

The crosslinkable nature of composite scaffold combined with the excellent integration between gelatin deposition and fibrous PCL suggests the possibility of preparing multilayered constructs by stacking and photocrosslinking the individual layers of composite scaffold. To test whether this approach could produce consistent integration between layered sheets, two layers were partially overlapped with or without subsequent photocrosslinking (Fig. 4 A). Without photocrosslinking, some of the scaffold pairs no longer adhered to each other after washing, whereas all crosslinked scaffold layers remain intact throughout the test (Fig. 4 B, areas between blue lines are overlapping regions). After 1 or 7 days of washing in PBS at 37 °C, unseparated scaffold pairs were securely mounted and subjected to uniaxial stretch force until separation of the scaffold layers. The load-displacement curve revealed markedly higher resistance to tension and higher maximum load in the crosslinked scaffold pair, indicating that the stacked layers of sheets can bond with each other (Fig. 4 C, D).

To create multilayered constructs, 5 sheets of scaffold in rectangular shape (8 mm x 4 mm) were wetted with photo-initiator solution, stacked and exposed to VL to crosslink adjacent scaffold layers (Fig. 5 A). The average thickness of constructs was 0.367 mm (n=12). After 7 days of washing in PBS, a substantial difference in scaffold integrity was identified by picrosirius red staining. As shown in Figure 5 B, without crosslinking (left, CN), the scaffold layers did not

adhere to each other due to mGLT loss. Sandwich constructs made from alternative layers of fibrous PCL and mGLT showed partial failure (center, PC), while crosslinked multilayer composite scaffolds remained fully integrated with negligible separation between layers (right, CC). Mechanical testing showed significantly higher tensile strength of crosslinked scaffolds (1.55 ± 0.49 MPa) than non-crosslinked scaffolds (0.92 ± 0.38 MPa, Fig. 5 C, CC vs. CN, $p < 0.05$, $n=6$), while the integration between layers exerted little impact on overall tensile strength (Fig. 5 C, PC vs. CC, $n=6$).

3.4. Cell incorporation and activity in multilayered construct

hASCs were impregnated into the crosslinked PCL/mGLT sandwich constructs (PC) or crosslinked composite constructs (CC) to generate a cell-laden graft. After 7 days of culture, EthD-1 staining for cells showed that most hASCs were localized in the interstitial space between adjacent layers (Fig. 6 A; red). In spite of the similarity in cell distribution, crosslinked composite constructs again demonstrated greater structural integrity than that of sandwich constructs, in which the individual scaffold layers had separated (Fig. 6 A; dark red). Moreover, hASCs impregnated in composite scaffold displayed significantly higher metabolic activity (Fig. 6 B), while total cell number, reflected by the content of dsDNA, remained comparable between two construct formats (Fig. 6 C). Taken together, these two findings suggest that constructs made from composite scaffolds promote cell metabolism but not proliferation.

3.5. Characterization of tendon phenotype induced by aligned multilayered constructs

To create tendon-mimicking constructs, aligned composite scaffolds were prepared and anisotropy confirmed by SEM (Fig. 7 A) and mechanical testing along planes perpendicular (Cross) or parallel (Longi.) to the orientation of fibers (Fig. 7 C, 0.12 ± 0.01 vs. 2.51 ± 1.33

MPa). F-actin fluorescent staining showed that impregnated hASCs adopted elongated morphology and were aligned in the direction of fibers (Fig. 7 B; green, F-actin; blue, DAPI), whereas no uniformity in orientation was noted in non-aligned scaffolds (Supplementary Fig. S3). For tendon cell lineage commitment, hASCs were treated with 10 ng/ml TGF- β 3 for 7 days. Real-time PCR assay showed pronounced upregulation of tendon markers SCX and TNC (Fig. 7 D), indicating that encapsulated cells remained responsive to soluble tenogenic factors, and that the construct possessed sufficient porosity for cells to receive exogenous biochemical cues.

4. Discussion

Scaffolds are of critical importance in the context of tissue engineering, serving to provide a physical substrate that mimics the *in vivo* milieu of healthy tissues and thus orchestrate the activity of therapeutic cells in a tissue-specific fashion [38]. In terms of tendon tissue engineering, a number of scaffold designs have been developed to reproduce one or multiple structural/compositional characteristics of tendon tissues, among which aligned, electrospun fibrous scaffolds and the 3D hydrogels have been frequently implemented in preclinical models due to their capability of presenting the topographical cues and native biochemical cues to seeded therapeutic cells, respectively [15, 16, 28, 39]. Therefore, use of a composite scaffold consisting of fibrous scaffold sheets and hydrogels is a potentially promising approach to mimic the physical and biological features of tendon tissue.

To achieve this goal, we first prepared PCL/mGLT composite scaffolds by dual electrospinning. Upon VL irradiation, the free radicals generated from the photo-initiator resulted in crosslinking via methacrylate on the gelatin backbone [40], thereby generating a hydrogel network within the PCL fiber mesh (Fig. 3 A). We chose gelatin rather than collagen as the bioactive building block

of the construct for two reasons: (1) as a product of denatured collagen [41], gelatin possesses a portion of the amino acid sequence and bioactive motifs of the parent collagen and is consequently chemically similar to native collagen chains; and (2) gelatin is far less costly compared to purified collagen (~25 USD/100 g vs. 650 USD/100 mg; pricing from Sigma-Aldrich) while more stable to organic solvent dissolution [42]. These features make gelatin suitable for industry-scale scaffold manufacturing.

For electrospinning, PCL and mGLT were dissolved and extruded separately to create intercalated PCL and mGLT fibers so that the mechanical strength of the PCL scaffold mesh is not compromised by the change in mGLT structure upon hydration. In contrast, electrospinning of a mixed solution of gelatin and PCL led to a collection of fibers comprised of alternating segments of gelatin and PCL, in which the dissolution of gelatin will presumably lead to fiber dissociation at gelatin regions and sequential loss of overall mechanical strength [43].

Concentrations of PCL and mGLT ranging from 10% to 22% were tested, and 18% PCL and 20% mGLT were chosen based on the stability of the solution stream during electrospinning [44]. However, improvement in tensile strength is clearly needed if the scaffold is intended to be used to construct a clinical tendon graft (~550 MPa) [45], for the ultimate tensile strength (UTS) of multilayered PCL-mGLT sandwich construct and composite construct was only 1.41 ± 0.15 MPa and 1.45 ± 0.19 MPa, respectively (n=6). **One possible strategy to improve mechanical strength would be the inclusion of textile patterns, such as braiding or weaving [46].**

Although mGLT fibers rapidly dissolved upon wetting and therefore possibly acted as sacrificial fiber to increase the pore size of the scaffold, we did not observe improved cell infiltration into the interior region of the crosslinked scaffolds (Supplementary Fig. S4) [47]. The crosslinked gelatin hydrogel formed within the PCL fiber mesh may occupy the pores and impede cell

migration. To address this potential issue, a peptide linker containing a matrix metalloproteinase (MMP)-sensitive motif might be incorporated to generate a more cell-cleavable hydrogel within a fibrous scaffold to improve cell infiltration [48, 49].

In addition to presenting multiple environmental cues, multi-layering of fibrous scaffold sheets can be employed to reconstitute the 3D architecture of a native tissue [38]. To achieve this goal, the rapid, robust integration between scaffold layers and sufficient incorporation of therapeutic cells must be carefully balanced [46, 50, 51]. In the scaffold described here, the gelatin component was found evenly distributed throughout the depth of PCL mesh, and therefore enabled integration of multiple sheets along the z axis upon photocrosslinking. However, the constructs used in this study were made of no more than five layers of scaffold sheet to ensure the orthogonal penetration of light into the construct for sufficient crosslinking. Higher degree of stacking may result in inadequate crosslinking in the center zone of the construct due to the accumulated opacity. To address such restriction on construct thickness, thermo-responsive or chemically initiated crosslinkers may be considered. The VL-based photocrosslinking technique employed in our study is biocompatible, as our previous study found less than 6% cell death in the encapsulated cell population [52]. By combining the two aforementioned characteristics, we have achieved a robust and instant integration of scaffold layers that is compatible with simultaneous, sufficient cell encapsulation.

For future study, the composite scaffold is open to further modifications that aim at eliciting the tenogenesis of impregnated cells. For instance, heparin may be conjugated to the gelatin backbone to sequester and subsequently deliver exogenous tenogenic growth factors into the constructs [53]. Tendon tissue derived-ECM may also be incorporated into the hydrogel portion of the constructs to recapitulate the proteinaceous microenvironment of native tendon tissue [28].

Moreover, given the inflammatory milieu of damaged tendons and the co-morbidities possible in patients, the response of native cells derived from diseased tendon tissues to the scaffold created in this study is worthy of future exploration in order to further elucidate the scaffold's potential as the biomaterial component of tendon implant. It is noteworthy that Hakimi et al. reported excellent attachment and alignment of tendon cells derived from chronic tendinopathy patients on the electrospun component of a layered electrospun and woven scaffold, along with retention of tenogenic cell phenotype [46]. Finally, we believe that the scaffold engineered in this study as a building block for multilayer constructs should have applications beyond tendon tissue engineering in the fabrication of tissue grafts that contain both a fibrous component and a hydrogel portion.

5. Conclusion

In this study, we have developed a novel composite scaffold consisting of fibrous PCL and methacrylated gelatin (mGLT) interspersed by dual-electrospinning. The crosslinkable nature of the composite scaffold, together with the excellent integration of the gelatin within the PCL mesh, allowed the creation of a multilayered construct as a tendon graft through photo-crosslinking of stacked scaffold sheets. Human ASCs were impregnated into the scaffold to generate a cell-laden construct and were seen to align along the orientation of the fibers. Seeded cells adopted tendon cell phenotype upon treatment with TGF- β 3. These findings provide information for the development of more advanced tendon grafts that can mimic both structural and cellular characteristics of native tendon tissues.

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Disclosure Statement

No competing financial interests exist.

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Figure Legends

Fig. 1. Composite scaffold preparation. Dual electrospinning was employed to fabricate a scaffold containing PCL and mGLT fibers (Insert 1). Dry scaffold was wetted with aqueous photo-initiator solution (Insert 2) and then photocrosslinked by visible light (VL) to retain the gelatin (Insert 3).

Fig. 2. Characterization of scaffold architecture. (A) Fluorescently labeled PCL (red) and gelatin (green) displayed a fibrous morphology and interspersed distribution within the dry composite scaffold prepared by dual electrospinning (Composite Dry). Wetting of the scaffold with an aqueous solution resulted in dissolution of the gelatin fibers (green), whereas the PCL fibers (red) remained intact (Composite Wet). PCL fibers alone showed no change with wetting. (B) SEM revealed distinct microscopic architecture of the composite scaffold, in which ribbon-like bands were seen in addition to the predominant cylindrical fibers (Composite Dry, indicated by arrow in insert). After wetting, adjacent fibers were bridged or wrapped with a sheath of gelatin (Composite Wet, indicated by arrows in insert). Unlike the composite mesh, scaffolds made of only PCL showed negligible differences in morphology before and after wetting (PCL Dry vs. PCL Wet).

Fig. 3. Examination of gelatin retention. (A) Picrosirius red staining showed that non-crosslinked scaffolds lost the majority of incorporated gelatin after washing (Composite non-crosslinked). In contrast, photo-crosslinked scaffolds retained a portion of incorporated gelatin within the scaffold mesh (Composite crosslinked, red). (B) Non-crosslinked scaffolds showed significantly

higher weight loss compared to crosslinked scaffolds. (C) Crosslinked scaffolds retained ~45% of their initial gelatin content based on hydroxyproline assay, while non-crosslinked scaffolds retained less than 3%. (D) Mechanical testing of crosslinked scaffolds demonstrated equivalent maximum load to that of dry composite scaffolds, while non-crosslinked scaffolds showed approximately 16% reduction after washing. *, $p<0.05$; **, $p<0.01$, $n=4$.

Fig. 4. Laminar integration of scaffold sheets. (A) Scaffold pairs were prepared by overlaying one scaffold sheet atop another sheet of identical rectangular shape, so that 1/3 of their lengths overlapped. The sheets were then wetted with photo-initiator and photocrosslinked. (B) Without crosslinking, some of the scaffold pairs fell apart after washing, whereas crosslinked scaffold pairs remained intact. Blue lines suggested the boundary of overlapping regions. (C) Representative load-displacement curve of crosslinked scaffold pairs showed higher resistance to separation. (D) Crosslinked scaffold pairs showed significantly higher maximum load before separation compared to non-crosslinked pairs. **, $p<0.01$; $n=6$.

Fig. 5. Preparation and characterization of a multilayer construct. (A) Scaffold sheets were wetted, stacked and exposed to VL for crosslink formation between adjacent scaffold layers to create a complex multi-layered structure. (B) Picrosirius red staining showed pronounced difference in scaffold integrity after washing among the three groups: stacked composite scaffold without crosslinking (CN, left), sandwich construct made from alternative layers of fibrous PCL and mGLT (PC, center), and crosslinked composite scaffold (CC, right). (C) Crosslinked scaffolds showed higher tensile strength than non-crosslinked scaffolds (CC vs. CN); the integration between fibrous scaffolds had little impact on overall tensile strength (CC vs. PC). *, $p<0.05$; $n=6$.

Fig. 6. Analysis of cell distribution and metabolic activity in multilayer constructs. (A) Ethidium homodimer-1 (EthD-1, red) staining indicated that most cells were localized between adjacent layers. Crosslinked composite constructs (CC) demonstrated evidently higher structural integrity than crosslinked sandwich constructs (PC). (B) MTS assay revealed significantly higher metabolic activity of cells impregnated in composite scaffold (CC), whereas the total cell number remained comparable to that of sandwich constructs (PC). *, $p < 0.05$; $n = 8$.

Fig. 7. Tendon-like features in cell-impregnated multilayer constructs. (A) Fiber alignment observed by SEM. (B) Elongated morphology of human adipose stem cells (hASCs) aligned in the direction of fibers (green, F-actin; blue, nuclei). (C) Anisotropy based on tensile strength properties measured by mechanical testing along two directions (Longi. vs. Cross). (D) Significant upregulation of tendon markers scleraxis (SCX) and tenascin C (TNC) upon treatment with exogenous tenogenic factor TGF- $\beta 3$, measured by real-time PCR analysis.

Supplementary Fig. S1. Synthesis and crosslinking of methacrylated gelatin (mGLT). (A) The amine groups on gelatin molecules were reacted with methacrylic anhydride to add methacrylate pendant groups. (B) To create a hydrogel network, mGLT was crosslinked by VL irradiation in the presence of photo-initiator.

Supplementary Fig. S2. Picrosirius red staining of scaffolds. In crosslinked composite scaffolds, gelatin was evenly distributed within the fiber mesh, suggesting excellent integration between gelatin and PCL fibers. In contrast, mGLT solution cast atop PCL scaffold aggregated on the surface of PCL fibers after crosslinking.

Supplementary Fig. S3. Morphology of ASCs cultured on non-aligned scaffolds. hASCs seeded on random scaffold (left, SEM) exhibited polygonal shape without uniformity in orientation (right, confocal microscopy; green, F-actin; blue, nuclei).

Supplementary Fig. S4. DAPI-stained cross-sections of ASC-seeded scaffolds after one week of in vitro culture. Dissolution of gelatin without crosslinking increased scaffold porosity and therefore improved cell infiltration (Composite non-crosslink) compared to cells seeded on PCL scaffold. However, retention of gelatin via crosslinking appeared to impede ASC migration into the interior region of scaffold (Composite crosslink). Blue, DAPI; scaffolds were outlined by white dash lines.

Figure 1

Figure 2

A

B

Figure 3

Figure 5

Figure 6

Figure 7

Table 1. Primer sequences of genes analyzed by real-time PCR

Gene		Primer sequence (5'-3')	Product size (bp)
18S rRNA	Forward	GTAACCCGTTGAACCCCAT	151
	Reverse	CCATCCAATCGGTAGTAGCG	
SCX	Forward	ACACCCAGCCCAAACAGA	65
	Reverse	GCGGTCCTTGCTCAACTTTC	
TNC	Forward	GGTGGATGGATTGTGTTCTGAGA	328
	Reverse	CTGTGTCCTTGTCAAAGGTGGAGA	